Chlorophyll, Chloroplast Ribosomal RNA, and DNA Are Reduced by Barley Stripe Mosaic Virus Systemic Infection

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ABSTRACT

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Barley stripe mosaic virus became systemic 3 days after inoculation of barley plants in the two-leaf stage. The first leaf to show mosaic was leaf 3, which was 4.5 cm long at 3 days after inoculation when it was invaded by virus. Since all of leaf 3 showed mosaic, the events leading to mosaic occurred during cell expansion and after cell division, which is limited to the basal centimeter of young cereal leaves. Molecular changes associated with mosaic formation included a 50% reduction in chloroplast ribosomal RNA and chlorophyll, expressed as amount per unit DNA, in young leaves with an acute stage of infection. Cytoplasmic ribosomal RNA was not reduced. The amount of DNA per barley leaf and leaf fresh weight were reduced as a result of systemic virus infection. Chloroplast ribosomal RNA was not detectable by density gradient centrifugation in infected areas of maize leaves. An RNA-dependent RNA polymerase associated with virus infection reached maximum concentrations in young systemically infected barley leaves coincident with high rates of virus replication and first detectable decreases in chlorophyll and chloroplast ribosomal RNA.

Barley stripe mosaic virus (BSMV) is a seed-borne, multicomponent, rod-shaped virus (2,17). In addition to a typical mosaic, it induces an increased mutation rate in maize (6,29) and shrunken seed with chromosomal abnormalities in barley (28). These symptoms are development related in that their appearance depends on infection of crucial stages of differentiating tissue. The increased mutation rate in maize apparently results from activation of naturally occurring controlling elements or transposons (6,25). However, this does not occur unless the plant is systemically infected when the tassel tissue is differentiating (29). Likewise, mosaic symptoms appear in leaves that develop after the plant is systemically infected and not in inoculated leaves (22). This dependence of symptom type on systemic infection of differentiating tissue leads to the hypothesis that virus infection may influence the expression of some plant genes important for differentiation.

Much more research has been conducted on the properties of viruses than on the changes in plants leading to symptoms or the mechanisms by which symptoms are caused. Most of the information on mechanism of disease induction has been obtained by research on dicots (21). It is important to understand the mechanism of symptom formation in monocots because of their importance as major crop plants. Furthermore, monocots have an advantage as an experimental system for studying mosaic formation because young leaves provide a gradation of cell age from base (youngest) to tip (oldest). Although nothing is known about the molecular mechanism of mosaic formation in barley infected with BSMV, there is some information on changes in infected plants. Infected leaves were reported to have reduced weight and chlorophyll (27). Ultrastructural investigations have shown abnormal chloroplasts and an association of virions with chloroplast membranes in early stages of cellular infection (9,13,19,23). Peripheral vesicles associated with chloroplast membranes in early stages of infection stained with antibodies to dsRNA (20). Little is known of the manifestations of infection at the molecular level, although Pring (26) reported decreased synthesis of both chloroplast and cytoplasmic ribosomal RNA (rRNA), and Suzuki and Taniguchi (30) reported evidence for reduction in chloroplast rRNA and appearance of a low molecular weight RNA of about 2s. White and Brakke (32) reported decreased synthesis of chloroplast-associated proteins.

The first leaves to show systemic symptoms of severe strains of BSMV are almost completely yellow as if every cell were infected. This acute stage is followed by a chronic stage of typical mosaic with mixed green and yellow islands. We have determined the stage of development when a leaf must become systemically infected if it is to develop mosaic, and we have measured the effects of viral infection on concentrations of rRNAs, chlorophyll, virions, DNA, and an RNA-directed RNA polymerase in relation to stage of symptom development and leaf age. Two of the earliest changes were an increase in the RNA-directed RNA polymerase and a decrease in chlorophyll and chloroplast rRNA.

MATERIALS AND METHODS

Viruses and host plants. Barley plants (*Hordeum vulgare* L. 'Larker') were inoculated in the two-leaf stage with the ND18 (27), Argentine Mild, or White-Leaf (22) strain of BSMV. Unless stated otherwise, results are those obtained with the ND18 strain. Maize (*Zea mays* L. 'Golden Cross Bantam') was inoculated in the two-leaf stage with the ND18 strain. Control plants were mock-inoculated with water. Plants were grown in a greenhouse at 25–30 C. All assays were of combined leaves from 10 to 30 plants. The first leaf to appear on a plant was called number one.

Virion, protein, chlorophyll, and nucleic acid assays. For rRNA assay, leaves were ground in a Virtis homogenizer in 18 volumes (ml/g) of 110 mM $(NH_4)_2CO_3$, 330 mM NH_4Cl, 11 mM ethylenediaminetetraacetic acid (EDTA), which had been adjusted to pH 9.3 with NaOH (5). The extract was filtered through cheesecloth, added to 2 volumes (ml/g) of 10% sodium dodecyl sulfate (SDS), and incubated 30 min at 25 C. Most proteins were precipitated as potassium dodecyl sulfate complexes by adding 5 M potassium acetate (pH 8) to a final concentration of 0.3 M, incubating in an ice bath for 20 min, and centrifuging at low speed (e.g., 10 min at 10,000 rpm; 11,000 *g* average RCF). Portions (50–200 μl, depending on leaf age) of the clarified nucleic acid extract were centrifuged in the Beckman SW60 rotor for 3.5 hr at 54,000 rpm (300,000 *g* average RCF) at 15 C through linear 7.5–30% (w/v) sucrose gradients in NaGPS buffer (50 mM Na_2HPO, 100 mM glycine, 300 mM NaCl, 1 mM EDTA, pH 9.4) (5). RNA concentrations were calculated from areas under peaks of ultraviolet absorbance scans of the gradients (4).

Duplicate portions of the clarified nucleic acid extract (1.5 ml each) were added to equal volumes of isopropanol, incubated 1 hr at −20 C, and centrifuged 20 min at 6,000 rpm. The pellets were suspended in 0.5 M perchloric acid for DNA assay by the diphenylamine method (8).

Virion concentrations were estimated from ultraviolet absorbance of zones obtained by density gradient centrifugation (4) of extracts of barley leaves in 10 mM sodium phosphate, 5 mM EDTA, pH 7.0. Extracts were incubated 1 hr at 20 C to allow ribosomes to degrade, clarified by low-speed centrifugation, and made 1% in Triton X-100 before density gradient centrifugation.

Because of the small amount of available tissue, virions in yellow areas of infected maize leaves were assayed by electrophoresis of SDS-complexed capsid protein in 12% polyacrylamide gels with buffers of Laemmli (18). Leaf tissue was ground in 90 mM Na_2HPO, 10 mM NaH_2PO_4, 10 mM EDTA with 1% Triton X-100, and centrifuged 1 hr at 40,000 rpm in a Ti50 Beckman rotor through a 1-cm layer of 20% sucrose. There was no preceding low-speed centrifugation. Pellets were heated in sample buffer (2.5 ml/g of leaves) and 4–20 μl was applied per gel lane. Gels were stained with Coomassie Brilliant Blue R-250 and scanned for absorbance at 570 nm. Bovine serum albumin (BSA) was used for concentration standards. Control experiments showed that low concentrations of BSA and BSMV capsid protein bound similar amounts of Coomassie Brilliant Blue 250. Inclusion of a low-speed centrifugation step before the high-speed centrifugation resulted in loss of some virus and did not significantly improve the gel pattern.

Chlorophyll was determined from absorbance at 663 and 645 nm of an extract in 80% acetone (1).

Isolation and assay of membrane-bound, RNA-directed RNA polymerase. Details of this procedure were described by White and Dawson (32). Briefly, leaves showing systemic symptoms (except where noted) were homogenized in buffer and filtered, and a membrane fraction (31,000 *g* pellet) was prepared. The standard assay mixture had 50 μl of enzyme preparation in 100 μl of 100 mM Tris-Cl (pH 8.0 at 30 C), 4 mM $MgCl_2$, 2.5 mM dithioerythritol, 12.5 mM $(NH_4)_2SO_4$, 20 μg/ml of actinomycin D, 0.1 mM each adenosine 5′-triphosphate, cytidine 5′-triphosphate, guanosine 5′-triphosphate, and 1 μM ^{3}H-uridine 5′-triphosphate. The reaction was initiated by adding enzyme and was terminated by transferring two 40-μl samples onto a 2.3-cm disk of Whatman 3 MM filter paper. The disks were washed (33), and radioactivity was determined as previously described (32).

Isolation and polyacrylamide gel analysis of RNA products from RNA polymerase. BSMV RNA was isolated from purified virions (16), and BSMV dsRNA was isolated from plants 5 days after inoculation (24). Scaled up RNA polymerase reaction mixtures (2–4 ml) were phenol extracted and the recovered RNA was analyzed on 3.0% polyacryamide gels as previously described (10,11).

RESULTS

Disease development. Barley plants inoculated with BSMV in the two-leaf stage showed systemic symptoms in the third leaf at 4 or 5 days after inoculation. Excision of inoculated leaves on a portion of the plants at daily intervals after inoculation showed that the virus systemically invaded 50% of the inoculated plants by 3 days after inoculation when the third leaf averaged 4.5 cm long. The first leaf to show symptoms, i.e., leaf 3, had acute symptoms and was almost completely yellow with few green islands. This leaf had approximately twice the virus concentration (about 300 μg/leaf or 0.5–1 mg/g fresh weight) of subsequent leaves which had chronic symptoms and usually 75–150 μg of virus per mature leaf. The amount of virus per leaf, with either a chronic or an acute infection, reached a maximum when the leaf attained its maximum weight (150–500 mg) and thereafter remained constant or decreased. The concentration of virus per unit fresh weight in immature leaves 4, 5, or 6 with a chronic stage of infection varied from 125 to 350 μg/g in a series of experiments, but in any one experiment was the same for immature leaves of all lengths.

Changes in DNA content per leaf. Virus infection reduced leaf weight of barley plants. To find if the weight reduction was due to smaller cells or fewer cells, the number of cells was estimated by measuring total DNA. The average DNA content per leaf of infected plants 3 wk after inoculation was 71, 83, and 65% that of uninfected leaves for leaves 3, 4, and 5, respectively. The average DNA content of uninfected leaves was 108, 147, and 168 μg per leaf for leaves 3, 4, and 5, respectively. The average weight of infected leaves 3, 4, and 5 was 73, 76, and 76%, respectively, of the weight of corresponding uninfected leaves. The average weight of uninfected leaves 3, 4, and 5 was 363, 434, and 353 mg, respectively. These values are averages from three experiments with a total of 47 randomly selected infected plants and a like number of randomly selected, mock-inoculated, uninfected plants. The leaves all had ligules, but leaf 5 probably had not reached its full weight.

Effect of BSMV infection on rRNAs of barley. The 16s and 18s rRNAs are not separated by sucrose gradient centrifugation. Therefore, the relative concentrations of chloroplast and cytoplasmic rRNAs were determined from areas of 23s (large chloroplast rRNA) and 28s (large cytoplasmic rRNA) peaks. Chloroplast 23s rRNA was not detectable by density gradient centrifugation in extracts of very young, white leaves, regardless of whether they were infected or not. The concentration of 23s rRNA increased as the leaf grew and became green. In mature, uninfected leaves, the chloroplast 23s rRNA was approximately equal in amount to cytoplasmic 28s rRNA. The concentration of total rRNA in milligram per gram fresh weight of leaf was highest in very young leaves (1–1.5 mg/g in uninfected leaves and 1.5–2 mg/g in infected leaves) and decreased as the leaf grew and aged. Calculated on a per leaf basis, the amount of rRNA increased as the leaf grew and reached a maximum (75–125 μg/leaf) just before the leaf reached its full weight and thereafter decreased.

The amount of 23s rRNA did not differ between infected and uninfected leaves in young, nearly white leaves that had barely detectable 23s rRNA. However, partially expanded, as well as full-size infected green leaves had less 23s rRNA than comparable uninfected leaves. The ratio of 23s/28s rRNA was less in infected leaves (with BSMV Argentine Mild) than in uninfected leaves in 24 of 33 comparisons of immature leaves of matched lengths. For these comparisons, partly expanded leaves 4 and 5 with chronic symptoms from plants at least 12 days after inoculation were selected. If the ratio were higher or lower in the infected leaves only because of random sampling effects, it should have been lower half of the time. The probability of it being lower

24 times out of 33 is only 0.005 if it is hypothesized that there is no difference between infected and healthy leaves. Therefore, the difference between uninfected and infected leaves is significant.

To find if the ratio of 23s/28s rRNA was less in the infected leaves than in the uninfected leaves because of decreased 23s rRNA or increased 28s rRNA, the concentrations of rRNAs were compared to the concentration of DNA. Leaves in the acute stage of the disease were used to accentuate the differences. The first leaf tissue to show symptoms was the basal 6–8 cm of the emerged portion of leaf 3. This portion of leaf 3 was collected from all plants in two randomly selected pots at 5, 6, and 7 days after inoculation. The entire leaf 3 was collected at 12 days after inoculation, when it had reached full size. The leaves (10 to 12 for each experiment) were combined and cut into centimeter-long segments, and random samples were assayed for rRNA, DNA, and chlorophyll (Fig. 1).

Chloroplast 23s rRNA content was about 50% of normal at 5–7 days after inoculation and remained low, while the 23s rRNA in uninfected leaves declined between 7 and 12 days (Fig. 1). Chlorophyll content of infected leaves was about 50% of normal. The amount of cytoplasmic 28s rRNA was not decreased by infection. Similar results were obtained with the White Leaf strain of BSMV except that values for chlorophyll and 23s rRNA were less at 12 days than at 7 days after inoculation.

Effect of BSMV infection on maize rRNA. First symptoms on maize leaves appeared about 2 wk after inoculation. The first symptomatic leaf did not exhibit more severe symptoms than later leaves as occurred in barley. Symptoms consisted of yellow spots or streaks up to a few millimeters across. The spots remained yellow as the leaf aged. Yellow areas were excised and assayed separately from the green areas.

Virus could not be detected by infectivity assay in extracts of green areas nor could viral capsid protein be detected by SDS gel electrophoresis of high-speed centrifugation pellets. Extracts of yellow areas infected more than 50% of barley assay plants at dilutions of 1:100 and contained 70% as much capsid protein as barley leaves with an acute infection. The green areas of infected leaves had as much chloroplast 23s rRNA as uninfected leaves, but yellow areas had insufficient 23s RNA to detect by density gradient centrifugation.

Time of appearance of RNA-directed RNA polymerase. To further correlate the time of virus replication with the time of the effects on chloroplasts, the time course of RNA-directed RNA polymerase was determined. The membrane-bound RNA polymerase in BSMV-infected tissue was similar to that in other virus-infected leaves (14,15) in its requirement for the four nucleoside triphosphates and Mg^{2+} ion for activity, and in pH and temperature optima. Enzyme activity was insensitive to DNase, actinomycin-D, and alpha amanitin (data not shown). When analyzed by gel electrophoresis, the in vitro product from uninfected plants was polydisperse, whereas that from infected plants had two high molecular weight products, the largest of which was partially sensitive to RNase (Fig. 2).

For these particular experiments, barley seeds were planted on 4 to 6 successive days. Primary leaves only were inoculated with BSMV 6 days after planting. Primary leaves were harvested from plants 2 to 6 days after inoculation. Second leaves, which were systemically infected, were harvested 4 to 8 days after inoculation of the primary leaves. Results of three independent experiments are shown (Fig. 3). RNA-directed RNA polymerase activity was very low in primary inoculated leaves with maximal activity approximately 4 days after inoculation. RNA-directed RNA

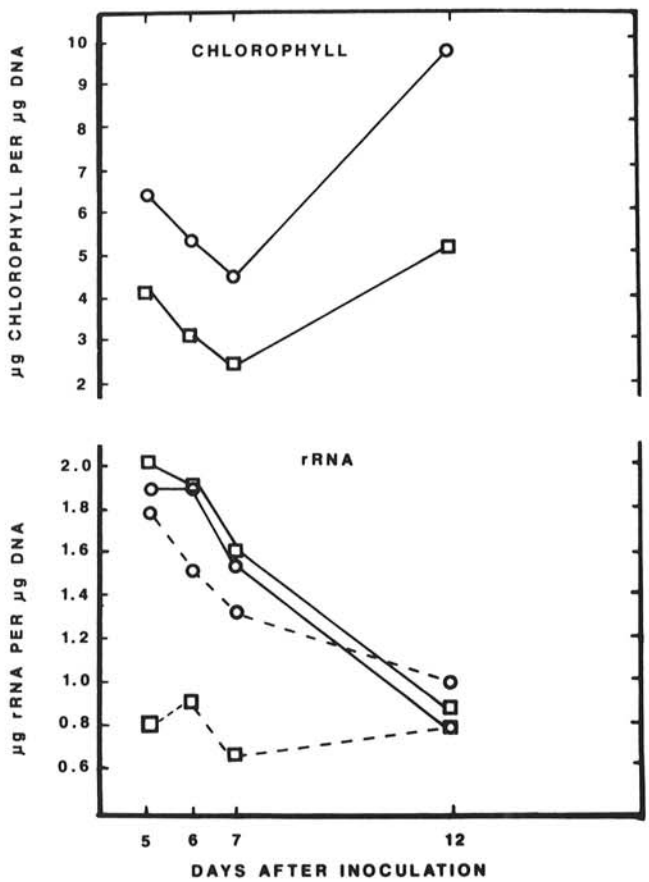

Fig. 1. Comparison of barley leaves infected with barley stripe mosaic virus (BSMV) (ND18) and uninfected, mock-inoculated barley leaves (leaf 3). Each point is an average of values from three experiments. In each experiment, leaf 3 was harvested from all the plants (10–12) in two randomly selected pots at 5, 6, 7, and 12 days after inoculation. Systemic symptoms first appeared on the basal exposed portion of leaf 3 at 5 days after inoculation. The basal 6-cm portion of leaf 3 was assayed for DNA, rRNA, and chlorophyll on days 5, 6, and 7, and the entire leaf was assayed on day 12. Leaf 3 was still expanding on days 5, 6, and 7 but was full grown with a ligule on day 12. The 23s rRNA is from chloroplast ribosomes and the 28s rRNA is from cytoplasmic ribosomes. Uninfected chlorophyll and 28s rRNA (o——o); infected chlorophyll and 28s rRNA (□——□); uninfected 23s rRNA (o- - -o); infected 23s rRNA (□- - -□).

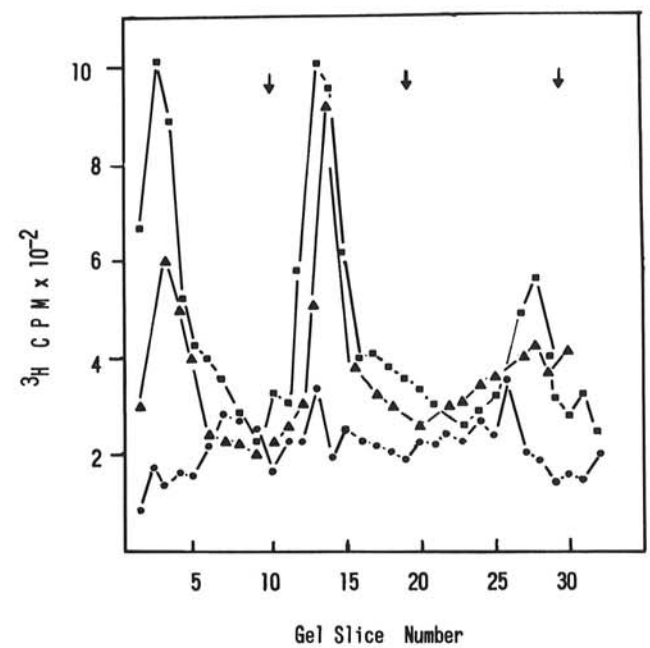

Fig. 2. Electrophoretic analysis on 3% polyacrylamide gels of the product of the membrane-bound RNA polymerase from barley infected with barley stripe mosaic virus (BSMV) (ND 18) and uninfected, mock-inoculated barley. Migration was from left to right. The arrows refer to positions of added internal standard brome mosaic virus RNAs (1.1 + 1.0 [unresolved], 0.7, and 0.26×10^6 Mr, respectively). Product from enzyme from BSMV-infected plants (■); product from BSMV-infected plant after RNase treatment at high salt (10,11) before counting (▲); and product from enzyme from uninfected plant (●).

polymerase activity was generally higher in systemically infected second leaves than in inoculated leaves reaching maximum activity 6 days after inoculation.

In older chronically infected leaves, the size of the leaf was used as an indication of age rather than days post inoculation. Generally the small leaves (less than 10 cm in length, still rolled) contained the highest RNA polymerase activity (Fig. 3). As the leaves expanded, the polymerase activity declined. Young No. 3 leaves had from 10-fold to 20-fold higher RNA polymerase activity than inoculated leaves. The fourth and fifth systemically infected leaves revealed RNA polymerase patterns similar to that of leaf 3 (data not shown).

DISCUSSION

The earliest detected cellular changes associated with systemic BSMV infection all involve chloroplasts. Lin and Langenberg (19,20) reported that the first detectable ultrastructural changes in infected root cells were an association of virions and vesicles with proplastids. We have found a marked decrease in chlorophyll and chloroplast rRNA as soon as symptoms are visible, concomitant with rapid accumulation of virus and a high concentration of RNA-dependent RNA polymerase. A decrease in chloroplast rRNA is probably associated with a decrease in chloroplast ribosomal proteins. A decrease in chloroplast nonribosomal proteins in BSMV-infected barley was reported earlier (31).

The fact that about a 50% decrease in chlorophyll and chloroplast rRNA was detected as soon as symptoms were visible suggests that changes may have started before symptoms were visible. This possibility has not been investigated, but moderate concentrations of virus have been found in leaf 3 before symptoms were visible (Brakke, *unpublished*).

Young leaves showed mosaic if they were 4–5 cm long when the plant became systemically infected. The entire young leaf showed mosaic by the time it was grown. Therefore, the events leading to mosaic did not occur at the stage of cell division because cells divide in the basal centimeter of immature cereal leaves (12). Rather, the events leading to mosaic occurred during cell maturation, a complex process involving division of proplastids and maturation of chloroplasts and synthesis of all the other cellular components. Development of chloroplasts requires coordinated expression of both nuclear and chloroplast genes (3,12). Each infected leaf synthesized a large amount of virus as it grew, in addition to its normal components. Studies on chronically infected leaves showed that, from the earliest stage of leaf growth at which they were detectable, chloroplast rRNAs and chlorophyll were present in lower concentrations per unit DNA in the infected than in the uninfected leaf. In contrast, infected leaves had as much cytoplasmic rRNA as uninfected leaves on a per unit DNA basis. Virus replication appears to either interfere with expression of genes essential to chloroplast development or lead to destruction of immature chloroplasts. The presence of normal levels of cytoplasmic rRNA shows that the virus infection did not suppress development of all cellular constituents.

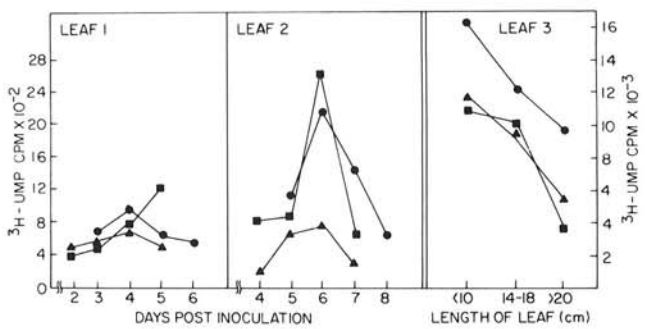

Fig. 3. Activity of the membrane-bound RNA polymerase from barley at various times after inoculation. Leaf 1 is the oldest leaf and was the only leaf inoculated. The length of leaf 3 is correlated with the time after inoculation. Results of 3 experiments.

Ideally, to detect changes at the cellular level, concentrations of cellular components should be expressed on a per cell basis. We think that DNA content is a reasonable alternative to number of cells determined by counting. We found DNA assays to be more reproducible than counts of chromic acid dispersed cells (7) and counts of nuclei from formaldehyde-fixed tissue. The amount of DNA per cell may not be exactly constant in infected and uninfected leaves because of possible changes in ploidy and, more likely, a possible decrease in chloroplast DNA. However, as a basis for comparison, DNA is preferable to alternatives such as dry weight or fresh weight.

The decrease in DNA content in infected leaves suggests that these leaves had fewer cells than uninfected leaves. The fresh weight was reduced by about the same amount in leaves 3 and 4 as the reduction in DNA. In leaf 5, fresh weight was reduced by a greater extent than DNA. Similar differences probably existed in dry weight because Pring and Timian (27) found no difference in percentage dry matter in Black Hulless barley leaves chronically infected with BSMV or in acutely infected leaves before onset of necrosis. Leaf 5 may have had smaller cells as well as fewer cells, whereas leaves 3 and 4 had fewer, but not smaller, cells.

The development of barley stripe mosaic is quite different in maize and barley. There is no sharp dividing line between yellow and green areas of infected barley leaves as there is in maize. The green areas of maize leaves appear to be uninfected. The green and yellow areas of barley leaves are too small to be excised and assayed separately as can be done with maize, but occasionally chronically infected barley leaves are found that have few yellow areas. Extracts of these leaves are highly infectious and virus is readily purified from them. Mosaic in barley appears to be due to presence of areas of the leaf with different intensities of infection, whereas in maize the yellow and green areas are infected and uninfected, respectively. Despite these differences, the virus-induced decrease in chloroplast components is common to both hosts.

Because mosaic is a development-related symptom, its formation is best studied in developing leaves. Selection of comparable infected and uninfected tissue is complicated by the almost continuous change in gene expression as a leaf grows and then ages. Selection of infected and uninfected leaves of equal sizes (as in one series of experiments reported above) may result in selection of infected cells that are older than the uninfected cells because of stunting due to the disease. In the case of the comparison of 23s/28s rRNA ratios, this would lead to higher values for the infected leaves because the 23s/28s rRNA ratio increases with cell age in young leaves. This is the opposite of what was found.

Pring (26) reported decreased incorporation of $^{32}PO_4$ in both 23s and 28s rRNAs in barley with an acute infection of BSMV, strain ND18. The cultivar he used, Black Hulless, is more susceptible than the cultivar Larker used here. In addition, he grew plants in a growth chamber with artificial lights, whereas we grew them in the greenhouse. Pring and Timian (27) found the highest concentration of the ND18 strain (342 μg/g) in the first leaf of Black Hulless barley to have chronic symptoms rather than in the acute stage. We found the highest concentration of ND18 and Argentine Mild strains (about 1 mg/g) in leaves of Larker barley with acute symptoms and only about half as much in leaves with chronic symptoms. Whether the differences in host cultivars and growing conditions are the sole reasons for the differences in results remains to be investigated. Our results agree qualitatively with those of Suzuki and Taniguchi (30), but quantitative comparison is complicated by instability of chloroplast 23s rRNA in gel electrophoresis by which they assayed RNA.

Successful investigation of the molecular interactions leading to mosaic development will depend on selection of tissue at the right stage of development. The results presented here and elsewhere (31) suggest that such tissue should be the greening areas of immature, expanding first or second systemically infected leaves. These leaves show decreased chlorophyll, chloroplast ribosomes, and chloroplast-related polypeptides, and rapid accumulation of virus and viral-specific RNAs and proteins. Use of the first leaves

to show symptoms is complicated by lack of synchrony from plant to plant in movement of virus to become systemic. However, with BSMV infection of barley, these first leaves are advantageous because thay are acutely infected and lack green islands. Infected Larker barley plants show increased tillering and delayed heading, which makes selection of tissue for meaningful comparison between uninfected and infected leaves more difficult in later stages of the disease.

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